REMARKS

Applicants respectfully request reconsideration of the present application.

1. Disposition of the Claims and Specification

Claims 44-62 are currently pending. Claims 44-51, 54, 58-59 and 61-62 are currently under consideration. Claims 52-53, 55-57 and 60 have been withdrawn. Claims 1-43 are canceled. Claims 44, 51 and 61-62 are amended. Support for the amendments to claims 44, 51 and 61-62 may be found in the specification, for example, at page 27, lines 18-23 and at page 29, line 7.

Because the foregoing amendments do not introduce new matter and are made to clarify the scope of the claims, entry thereof by the Examiner is respectfully requested. Applicants believe that the amendment places the application in condition for allowance.

2. Claim Rejections – 35 U.S.C. §§ 101 and 112, first paragraph

Claims 44-51, 54, 58-59 and 61-62 are rejected under 35 U.S.C. § 101 for the reasons of record set forth in the previous office action. Applicants previously argued that the claimed protein has a function with biological significance based on Sugiomto *et al.* and Hsu *et al.* Specifically, applicants argued that the claimed protein (which is identical to p34SEI1 and TRIP-Br1) antagonizes p16INK4a, which inhibits CDK4 and CDK6, as well as regulates E2F-1/DP-1 transcriptional activity.

The examiner responded by saying that this specific function, described in the post-filing date articles, is not recited in the instant specification. The examiner reasons that there "is no demonstration of the biological function of the instant protein" in the specification; however, the examiner does admit that the specification provides "an assertion that the protein has homology to cell cycle regulation proteins (page 3, lines 2-28)." Office Action at 3. The examiner further admits that the specification discloses that "expression of CECRP is closely associated with cell proliferation." Office Action at 3. The examiner asserts, however, that "bleach or gasoline are reagents that can affect cell proliferation." Office Action at 3.

In referring to the list of diseases recited on page 40 of the specification, the examiner further reasons that Applicants "have failed to recite a specific and substantial utility for this particular protein because Applicants have failed to disclose a nexus between the expression of the claimed protein and any of these conditions" ranging from "cirrhosis, heart disease and infections." Office Action at 3. Therefore, Applicants are claiming diagnosis of unrelated diseases using the polynucleotide encoding the instant protein." Office Action at 3. The examiner concludes by stating that because Applicants have not established "any connection or correlation of the asserted protein with any particular disease or disorder, the assertions cannot be considered credible." Office Action at 3.

The Claimed Protein is Useful in the Diagnosis of, Treatment and/or Prevention of Ovarian Cancer

Applicants respectfully disagree with the examiner. As the examiner has already pointed out, the specification discloses that the claimed CECRP protein is associated with cell proliferation by virtue of the fact that it is a cell cycle regulation protein. See page 3, lines 6-25. Accordingly, the examiner's assertion that such utility and function is non-specific because bleach and gasoline are two of many examples of reagents that can affect cell proliferation is unfounded.

Applicants further disagree with the examiner's statement regarding the lack of nexus between expression of the claimed CECRP protein and "any of the[] conditions" recited on page 40 of the specification. As explained above, Applicants have asserted that the claimed CECRP protein is associated with cell proliferation. As such, the expression of the claimed protein would specifically relate to the types of cancer listed in the specification at page 40, lines 7-11.

As further support, Applicants also refer to Tang *et al.*, "Identification of a Candidate Oncogene SEI-1 within a Minimal Amplified Region at 19q13.1 in Ovarian Cancer Cell Lines", CANCER RESEARCH 62: 7157-7161 (2002), attached as Exhibit 1. This article confirms that SEI-1 is "associated with cell proliferation and cell cycle control." Tang *et al.* at page 7159. The article also describes the finding that SEI-1 is overexpressed in ovarian

cancer cell lines and that it is a candidate oncogene with a role "in the development and progression of ovarian cancer." Tang *et al.* at pages 7159-60. Along these lines, Applicants note that the instant specification discloses the use of the claimed protein in the treatment or prevention of ovarian cancer (page 27, lines 24-25 and 30-31) as well as in the diagnosis of ovarian cancer (page 40, lines 2-3 and 10). Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection.

3. Claim Rejections – 35 U.S.C. §§ 101 and 112, first paragraph

The examiner has also rejected claims 44-51, 54 and 58-59 and 61-62 under 35 U.S.C. § 112, first paragraph because the claimed invention is not supported by either a substantially asserted utility or a well established utility, for the reasons set forth with respect to 35 U.S.C. § 101.

Applicants respectfully disagree with the examiner. Applicants have established a specific and substantial asserted utility, as described above in Section 2. Therefore, Applicants respectfully request withdrawal of this rejection.

4. Claim Rejections – 35 U.S.C. § 112, first paragraph

Claims 44, 51 and 61-62 are rejected by the examiner under 35 U.S.C. § 112, first paragraph. The examiner asserts that the "at least 95% identical" limitation "still fails to adequately describe and enable an isolated protein that is at least 95% identical to the polypeptide of SEQ ID NO: 3." Office Action at 5. The examiner reasons that "Applicants do not teach which regions of said polypeptide are critical to encode a functional polypeptide." March 18, 2004 Office Action at 7. The examiner further states that the "specification does not provide the requisite examples ... that would allow the skilled artisan to produce a polypeptide having at least [95%] sequence identity of SEQ ID NO: 3, nor does the disclosure provide criteria that explicitly enable such critical features." March 18, 2004 Office Action at 7.

Applicants respectfully disagree with the examiner. Table 2 of the instant specification recites various structural and functional fragments of SEQ ID NO: 3 such as (1)

potential phosphorylation sites (at S44, S60, S98, S117, S123, S180 and T73) and (2) identifying sequences and/or structural motifs such as the inhibin beta chain signature sequence. Applicants believe that Table 2 accordingly discloses those structural elements that are critical to encoding a functional polypeptide. As such, a skilled artisan would be able to create functionally equivalent 95% identical polypeptides by following the teachings of the specification. Specifically, by using the information from Table 2, one of ordinary skill would know to retain those portions of the sequence identified in Table 2 when creating a variant 95% identical to SEQ ID NO: 3. Applicants respectfully request reconsideration and withdrawal of the rejection.

5. Claim Rejections – 35 U.S.C. § 112, second paragraph

Claims 44-51, 54, 58-59 and 61-62 are rejected under 35 U.S.C. § 112, second paragraph. The examiner reasons that the metes and bounds of the term "cell cycle regulating activity" are unclear. The examiner also states that the term "regulates cell proliferation" is indefinite because it is unclear whether the claimed polypeptide attenuates or exacerbates cell proliferation.

Applicants respectfully disagree with the examiner. Applicants believe that the term "regulates cell proliferation" is clearly defined in the specification so that a person of ordinary skill in the art would understand the metes and bounds of the term. *See*, e.g., page 27, lines 18-23, and at page 29, line 7, where it states that CECRP can be "an inhibitor of cell proliferation" as well as "a promoter of cell proliferation." However, to expedite prosecution, Applicants have amended claims 44, 51 and 61-62 to clarify that the claimed polypeptide "promotes or inhibits cell proliferation." Support for the amendment to claims 44, 51 and 61-62 may be found in the specification, for example, at page 27, lines 18-23 and at page 29, line 7. Applicants respectfully request reconsideration and withdrawal of the rejection.

6. Conclusion

Applicants believe that the present application is in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

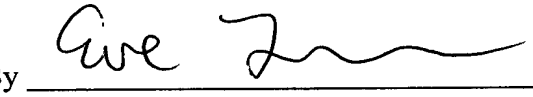
It is acknowledged that the foregoing amendments are submitted after final rejection. However, because the amendments do not introduce new matter or raise new issues, and because the amendments either place the application in condition for allowance or at least in better condition for appeal, entry thereof by the Examiner is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 10/11/04

By 

FOLEY & LARDNER LLP
Customer Number: 22428
Telephone: (202) 945-6142
Facsimile: (202) 672-5399

Eve L. Frank
Attorney for Applicant
Registration No. 46,785

[CANCER RESEARCH 62, 7157–7161, December 15, 2002]

Advances in Brief

Identification of a Candidate Oncogene *SEI-1* within a Minimal Amplified Region at 19q13.1 in Ovarian Cancer Cell Lines[1]

Terence C-M. Tang, Jonathan S. T. Sham, Dan Xie, Yan Fang, Ke-Ke Huo, Qiu-Liang Wu, and Xin-Yuan Guan[2]

Department of Clinical Oncology, The University of Hong Kong, Hong Kong, China [T. C-M. Y., J. S. T. S., X-Y. G.]; Department of Pathology [D. X.], and Cancer Institute [Y. F., Q-L. W.], Sun Yat-Sen University, Guangzhou, China; and State Key Laboratory of Genetic Engineering, Institute of Genetics, Fudan University, Shanghai, China [K-K. H.]

Abstract

High-level amplification of DNA sequence at 19q13.1 is one of the frequent genetic alterations in ovarian cancer. In an attempt to verify the minimal amplified region (MAR) at 19q13.1 and to identify the target oncogenes, 49 probes within a region from *D19S425* to *D19S907* (~19.5 cM) were used to survey the amplification status in four ovarian cancer cell lines that have been confirmed as containing amplification at 19q13.1. Two separated overlapping MARs, MAR1 (~200 kb) and MAR2 (~1.1 Mb), were identified at 19q13.1. Two candidate oncogenes, *AKT2* and *SEI-1*, were identified in MAR2. Amplification and overexpression of these two genes in four ovarian cancer cell lines were confirmed by Southern and Northern blot analyses. The proliferation-related function of *AKT2* and *SEI-1* suggests that both genes are likely to be biological targets of an amplification event at 19q13.1 in ovarian cancer and to play important roles in ovarian tumorigenesis.

Introduction

Ovarian cancer is the leading cause of death from female gynecological malignancies in developed countries, and its incidence has been increasing recently in Asian countries such as China and Singapore (1). Because of its insidious onset, the disease is diagnosed in 70% of cases in an advanced stage. Like other solid tumors, the development of ovarian cancer is also considered as a long-term process that involves multiple genetic alterations. Frequent genetic alterations including the gains of 1q, 3q, 8q, 17q, 19q, and 20q and frequent losses of 4q, 13q, 16q, 17p, and 18q have been detected in ovarian cancer by CGH[3] studies (2, 3).

Gene amplification and the consequent overexpression of the amplified oncogene is one of the common genetic alterations in various solid tumors that have been shown to play an important role in tumor pathogenesis, probably because the overexpression of the oncogene confers a growth advantage. Therefore, to identify commonly amplified chromosomal regions and the target oncogene within the regions it is imperative to understand the molecular mechanisms of the development and progression of cancer. In our previous study, the gain of 19q was detected in 12 (39%) of 31 primary ovarian cancers (3). High copy-number amplification of 19q13.1-q13.2 in the form of a hsr has been observed in four ovarian cancer cell lines (UACC326, UACC1123, UACC2727, and OVCAR-3) by chromosome microdissection (4). Although *AKT2* has been implicated as a candidate oncogene at 19q13 (5), coamplification of two or more oncogenes within one amplicon has been described previously. For example, chromosomal region 12q13-q15 has been shown to contain several genes, including *MDM2*, *CDK4*, *GLI*, *SAS*, and *CHOP*, that are potentially relevant to tumor growth. Coamplification and overexpression of *CDK4*, *SAS*, and *MDM2* has been frequently detected in human parosteal osteosarcomas (6). In addition, amplification of *AKT2* was only detected in about 12–13% of primary ovarian cancers in a previous study (5). Therefore, it is highly conceivable that one or more flanking oncogene(s) residing on either side of *AKT2* could also contribute to the development of ovarian cancer. In the present study, we have applied the physical mapping strategy by using FISH and Southern Blot analysis to identify the overlapping MARs at 19q13.1-q13.2 in the above mentioned four ovarian cancer cell lines. The results showed that there are two separate MARs at 19q13.1, each of which may contain one or more oncogenes involved in the amplification events in ovarian cancer.

Materials and Methods

Cell Lines and DNA Isolation. Ovarian cancer cell lines, UACC326, UACC1123, and UACC2727 were obtained from the Tissue Culture Core Service of the University of Arizona Comprehensive Cancer Center. Ovarian cancer cell line OVCAR3 was obtained from the American Type Culture Collection. Genomic DNA was extracted by a proteinase K/SDS digestion followed by phenol/chloroform/isoamyl alcohol extraction.

Probe Selection. About 200 STS markers within the interested region of 19q13.1-q13.2 (from *D19S425* to *D19S907*) were used as the initial starting point for probe selection. The genetic distance between *D19S425* and *D19S907* is about 19.5 cM (58.1–77.6 cM; GeneMap'99, NCBI). The sequences of STS markers were obtained from GeneMap'99, and BLAST search was performed to select corresponding cosmid clones for FISH study. Thirty cosmid clones were selected for FISH analysis and the distance between each other was ~1 cM. All of the cosmid clones were kindly provided by the Human Genome Center at Lawrence Livermore National Laboratory as free gifts. Thirteen known genes, one EST, and five unique genomic DNA sequences within the target region were selected to fill in gaps that could not be covered by cosmid clones. All of the cosmid clones and genes used in the present study are listed in Table 1.

FISH. Metaphases from four ovarian cancer cell lines were prepared for FISH analysis by a standard method. FISH was performed as described previously (7). Briefly, 200 ng of purified cosmid DNA were labeled with Biotin-14-dCTP using BioPrime DNA labeling system (Life Technologies, Inc., Rockville, MD). About 100 ng of biotinylated probe was mixed in 10 μl of hybridization mixture (containing 55% formamide, 2× SSC, and 1 μg human Cot I DNA) which was denatured at 75°C for 5 min. The slide containing metaphases from tumor cells was denatured in 70% formamide, 2× SSC at 70°C for 2 min and hybridized with the denatured probe at 37°C overnight. After wash, the hybridization signal was detected by two layers of FITC-conjugated avidin (Vector Laboratories, Burlingame, CA) and amplified with one layer of anti-avidin antibody (Vector Laboratories, Burlingame, CA). The slides was counterstained with 0.5 μg/ml DAPI in an antifade solution and examined with a Zeiss Axiophot microscope equipped with a dual bandpass filter.

Received 7/15/02; accepted 10/30/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by the Leung Kwok Tze Foundation and Chinese Visiting Scholar Foundation of Key Lab in University.

[2] To whom requests for reprints should be addressed, at Department of Clinical Oncology, The University of Hong Kong, Room 109, School of Chinese Medicine, 10 Sassoon Road, Hong Kong, China; Fax: 852-28169126; E-mail: xyguan@hkucc.hku.hk.

[3] The abbreviations used are: CGH, comparative genomic hybridization; hsr, homogeneously staining region; MAR, minimal amplified region; FISH, fluorescence *in situ* hybridization; DAPI, 4′,6-diamidino-2-phenylindole; STS, sequence tagged site; NCBI, National Center for Biotechnology Information.

Table 1 *Summary of amplification pattern at 19q13.1 in four ovarian cancer cell lines*[a]

MARs (MAR1, from sts-N29627 to Bda24b07; MAR2, from SGC32767 to sts-N31928) are shown in bold.

STS	PD (kb)	Clone/Gene	Test	326	1123	2727	OVCAR-3
sts-H96891	35,449	*KIAA0355*	Southern	−	−	−	−
stSG44812	35,609	DNA	Southern	+	+	+	−
stSG45410	36,053	DNA	Southern	+	++	+	−
stSG53555	36,333	F19410	FISH	−	−	−	−
stSG30207	36,366	R30879	FISH	−	−	−	−
WI-15623	36,422	F24108	FISH	++	++	++	+
sts-N29627	**36,677**	**R28461**	**FISH**	+++	+++	+++	+++
stSG54840	**36,755**	**R31396**	**FISH**	+++	+++	+++	+++
EST209638	**36,817**	**R30072**	**FISH**	+++	+++	++	++
Bda24b07	**36,884**	**F25965**	**FISH**	+++	+++	+++	++
stSG48421	36,930	F19541	FISH	+	+	+	−
A006G29	37,034	F19399	FISH	+	+	++	+
SHGC-8726	37,049	R31874	FISH	−	+	+	−
stSG30073	37,139	R33743	FISH	++	+++	++	−
stSG4652	37,165	R31491	FISH	++	+++	++	−
WI-12306	37,255	F24590	FISH	+	++	++	−
SHGC-11909	37,280	R33853	FISH	+	++	−	−
WI-9312	37,436	*OZF*	Southern	++	+	−	+
stSG3271	37,713	*KIAA1615*	Southern	+	−	−	−
stSG10281	38,057	EST	Southern	++	+	+	+
stSG43885	38,517	DNA	Southern	+	++	++	+
stSG49419	38,772	DNA	Southern	+	++	+	+
A007F08	39,397	*KOP*	Southern	+	++	+	+
stSG46747	39,476	F5960	FISH	+	++	++	+
stSG38855	39,693	F24108	FISH	+	++	+	+
sts-T78012	40,018	*SerRSmt*	Southern	+	++	+	−
stSG35865	40,265	*MLK2*	Southern	+	+	+	+
stSG12757	40,412	F23149	FISH	+	++	+	−
SGC32767	**40,434**	***GMFG***	**Southern**	+++	+++	++	++
sts-AA024814	**40,491**	**F23149**	**FISH**	+++	+++	+++	+++
U56402	**40,600**	***SUPT5H***	**Southern**	+++	+++	+++	+++
sts-R31571	**40,739**	**F22105**	**FISH**	+++	+++	+++	+++
SHGC-34102	**40,841**	**R30692**	**FISH**	++	+++	++	++
stSG26887	**40,995**	**R30669**	**FISH**	++	+++	++	++
SGC30851	**41,400**	***AKT2***	**Southern**	+++	+++	+++	++
sts-N31928	**41,568**	***SEI1***	**Southern**	+++	+++	+++	++
SHGC-111828	41,762	DNA	Southern	−	+	+	−
stSG12762	42,426	*TKR*	Southern	−	−	−	−
stSG50575	42,682	F6697	FISH	−	−	−	−
stSG41396	42,765	R32065	FISH	−	−	−	−
WI-17429	43,658	F19434	FISH	−	−	−	−
U06673	43,813	F24083	FISH	+	−	−	−
SHGC-11451	43,958	F19987	FISH	++	−	−	−
R76259	44,057	F21645	FISH	++	−	+	−
SGC34531	44,770	F22162	FISH	+	−	−	−
stSG26040	44,881	F17127	FISH	+	−	−	−
sts-W49696	45,259	R29827	FISH	−	−	−	−
stSG31317	45,652	*CBL-3*	Southern	−	−	−	−
WI-14251	49,359	*KIAA0955*	Southern	−	−	−	−

[a] −, no amplification; +, low-level amplification; ++, medium-level amplification; +++, high-level amplification.

Southern and Northern Blot Analyses. Southern blot hybridization was performed by a standard method. The PCR products of cDNA and unique genomic DNA sequences were labeled with [^{32}P]dCTP using Random Labeling kit (Life Technologies, Inc., Rockville, MD). Fifteen μg of genomic DNA isolated from ovarian cancer cell lines were digested with *Eco*RI, fractionated on 1% agarose gel, transferred to a nylon membrane (Bio-Rad, Hercules, CA), and hybridized overnight at 42°C with ^{32}P-probes. Ten μg of total cellular RNA, prepared by Trizol/chloroform method, were size fractionated on 1% agarose/2.2 M formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-probes.

Tissue Microarray. A total of 200 epithelial ovarian cancer cases with 400 tissue specimens were obtained from the archives of Cancer Center, Sun Yat-sen University of Medical Sciences, Guangzhou, China. Two specimens, one from tumor tissue and the other from surrounding nontumor tissue, were selected. The tissue microarray was constructed as described previously (8). Briefly, tissue cylinders with a diameter of 0.6 mm were taken from the selected regions of a donor block and then precisely punched into a recipient paraffin block using a tissue-arraying instrument (Beecher Instruments, Silver Spring, MD). Five-μm consecutive sections of the microarray block were made with a microtome.

Three cosmid clones, R31396 (mapped within MAR1), F23149 (within MAR2), F19514 (between the two MARs), were selected as FISH probes. The FISH reaction was performed as described previously (7) with some modifications. Briefly, the tissue array section was deparaffinized, treated with protenase K (400 μg/ml) at 37°C for 45 min, denatured at 75°C in 70% formamide, 2× SSC for 5 min, and hybridized with biotin-labeled cosmid probe at 37°C for 2 days. Washing condition and posthybridization treatment were identical as described above.

Results

Identification of Two MARs. Thirty cosmid clones and 19 DNA sequences, which cover a genetic region of 19.5 cM (from *D19S425* to *D19S907*) at 19q13.1-q13.2 were used to define the overlapping MAR among the four ovarian cancer cell lines by FISH and Southern hybridization, respectively. All four of the ovarian cancer cell lines used in this study were aneuploid and displayed chromosome numbers in the range of near-triploid except UACC1123, which was near-diploid. The degree of DNA amplification at 19q13.1 was divided into three levels according to the number of FISH signals and DNA amplification-fold detected by Southern hybridization, respectively. Low-level amplification (+) was defined as 2–5 extra FISH signals or 1–2-fold amplification of tested DNA sequences detected by FISH or Southern blot hybridization. Medium-level amplification (++) was defined as detection of 6–10 extra FISH signals or 2.5–4-fold ampli-

fication of tested DNA sequence. High-level amplification (+++) was defined as the detection of more than 10 extra FISH signals or 5-fold or more of amplification of tested DNA sequence. In this study, FISH signals in both metaphase chromosomes and interphase nuclei were counted. The extra FISH signals were determined based on the ploidy of each cell line. The amplification patterns of hsr detected by FISH and CGH in four ovarian cancer cell lines are shown in Fig. 1.

Analysis results showed that the amplicons at 19q13.1 were not continuous in all four of the tested ovarian cancer cell lines, and two separate overlapping MARs (MAR1 and MAR2) were identified. The order of the STS markers corresponding to the cosmid clones and known genes were determined by searching NCBI Map Viewer (dated on August 30, 2002). MAR1 is ~207 kb (between 36,677 and 36,884 kb) and MAR2 is ~1124 kb (between 40,434 and 41,568 kb). The distance between MAR1 and MAR2 is ~3.5 Mb. The results were summarized in Table 1.

Overexpression of *AKT2* and *SEI-1*. Candidate oncogenes within the MARs were screened through database search with NCBI Map Viewer. Several known genes were found in both MARs, including *SUPT5H*, *SEI-1*, *GMFG*, and *AKT2* in MAR2. Amplification of these genes had been detected by Southern blot hybridization, and examples of amplification of *AKT2* and *SEI-1* are shown in Fig. 2*A*. Among these genes, *AKT2* and *SEI-1* have been associated with cell proliferation and cell cycle control. Therefore, the RNA expression levels of these two genes were studied by Northern blot analysis to determine whether they were overexpressed in the tested ovarian cancer cell lines. The result showed that both *AKT2* and *SEI-1* were indeed overexpressed in all four of the ovarian cancer cell lines comparing with the total RNA from normal ovary (Fig. 2*B*).

Tissue Microarray. A tissue microarray section containing 400 specimens from 200 primary ovarian cancers was used to survey the amplification frequency of MAR1 and MAR2 at 19q13.1 in primary ovarian cancers. FISH hybridization was performed using cosmid clones R31396, F23149, and F19514 from MAR1, MAR2, and the low-amplified region between two MARs, respectively. FISH signals in 50 interphase nuclei were counted for each case. Informative FISH signals were observed in 103 of 200, 96 of 200, and 98 of 200 cases detected by R31396, F23149, and F19514, respectively. The noninformative samples might be caused by the missing of or unrepresentative tumor samples in the array section or failure of the detection. Amplification of DNA sequence in MAR1, MAR2, and region between the two MARs was detected in 17 (17%) of 101, 19 (20%) of 96, and 9 (9%) of 98 primary ovarian cancers, respectively (Fig. 3).

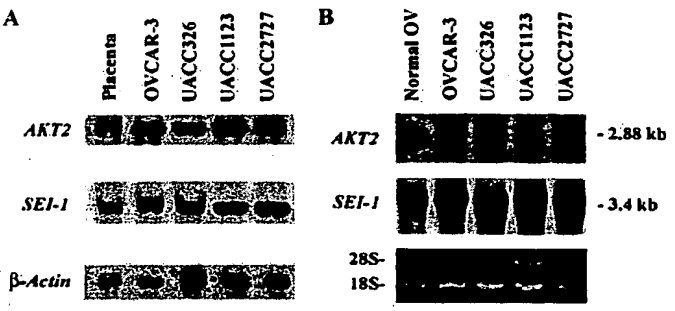


Fig. 2. Amplification and overexpression of *AKT2* and *SEI-1* in four ovarian cancer cell lines. In *A*, Southern blot analysis demonstrates amplification of *AKT2* and *SEI-1* in four ovarian cancer cell lines. A probe for *β-actin* was used for loading control. *B*, overexpression of *AKT2* and *SEI-1* in ovarian cancer cell lines was detected by Northern blot analysis. Control RNA was from normal ovary. 28S RNA band stained with ethidium bromide for the Northern blot was used as loading control.

Discussion

Amplification of 19q13.1-q13.2 is one of the common genetic alterations in ovarian cancer, and overexpression of putative oncogene *AKT2* at 19q13.1, which encodes a serine/threonine kinase, has been associated with the pathogenesis of ovarian cancer. Amplification of 19q13.1-q13.2 was also frequently detected in gastric cardia adenocarcinoma (9) and primary pancreatic carcinoma (10). These studies strongly indicate that 19q13.1-q13.2 may contain an oncogene(s) related to the pathogenesis and progression of various solid tumors including ovarian cancer.

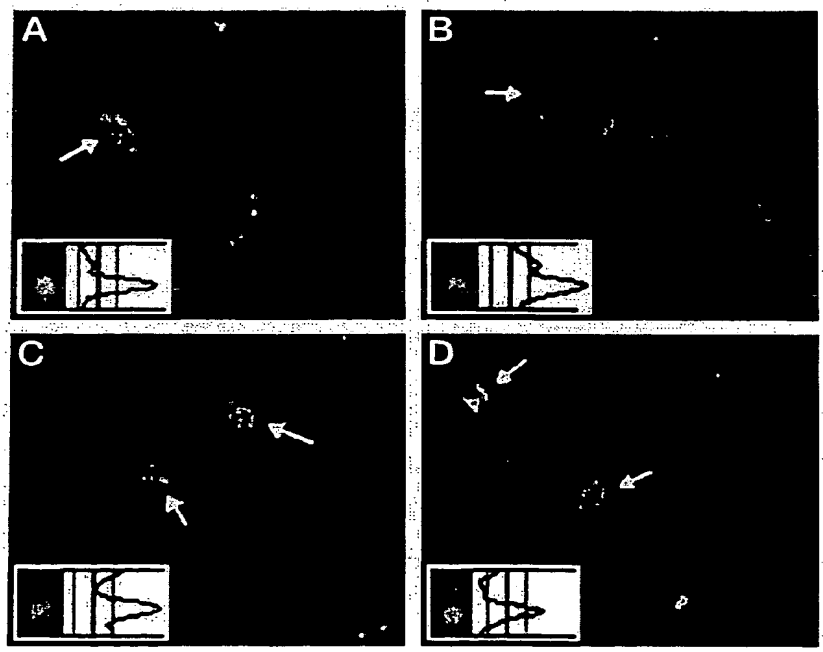


Fig. 1. FISH of cosmid clone (F23149) within MAR2 to metaphase spreads containing hsr markers in ovarian cancer cell lines UACC326 (*A*), UACC1123 (*B*), UACC2727 (*C*), and OVCAR-3 (*D*). Chromosomes (partial metaphases) were conterstained with DAPI (*blue*) and FISH signals (*green*); *white arrows*, hsr regions. *Inset*, the amplification of 19q13.1-q13.2 in each cell line detected by CGH.

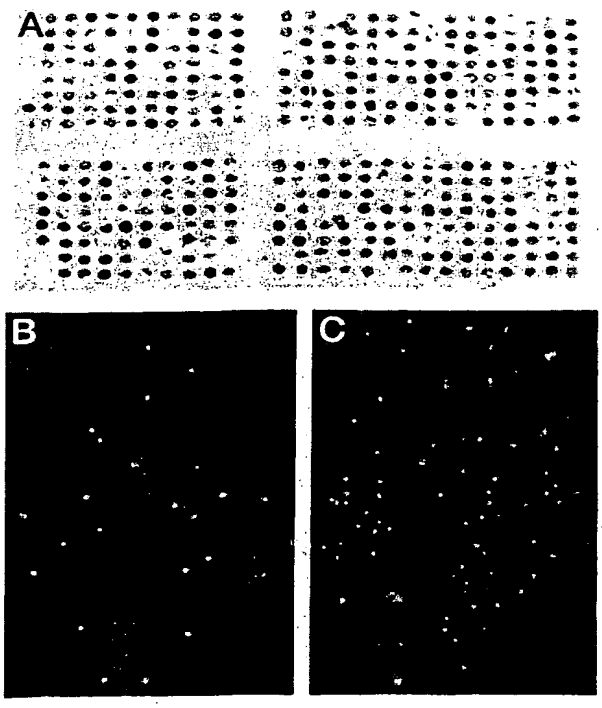


Fig. 3. Tissue array for FISH analysis. *A*, overview of H&E-stained sections of tissue array containing 400 specimens from 200 primary ovarian cancers. *B* and *C*, two examples of amplification of DNA sequence within MAR2, detected with cosmid probe F23149. About 6–8 and >10 green signals were detected in case 71 (*B*) and case 122 (*C*), respectively. The interphase nuclei were conterstained with DAPI.

In this study, four ovarian cancer cell lines containing amplicon of 19q13.1-q13.2 in the form of hsr were characterized to define the overlapping MAR. Forty-nine cosmid clones and DNA markers within a 13.9-Mb region (average distance between two markers is ~280 kb) at 19q13.1 were selected to detect overlapping MARs. The resolution of markers selected in the critical region from stSG53555 to stSG12762 (36,333–42,426 kb; NCBI Map Viewer) is ~170 kb. The result showed that amplicons in all four cell lines were not continuous, and two separate overlapping MARs were identified. Our tissue microarray study also supported the observation that the amplification frequencies of MAR1 and MAR2 were obviously higher than those in the region between MAR1 and MAR2. The fact that a hsr can be composed of two or more separated DNA sequences from the same or different chromosomes has been demonstrated by chromosome microdissection in breast cancer (11). In nine breast cell lines, 12 of 15 hsr markers were demonstrated to be composed of multiple chromosome components (11). Other studies also observed extensive DNA rearrangements in N-myc amplicons in neuroblastoma (12). These results indicate that the mechanism of hsr formation is very complex and might be associated with genomic instability.

The coamplification of two separated MARs in all four tested ovarian cancer cell lines suggests that the coamplification is not random. It is highly possible that the target genes located in these two regions may provide a stronger selective advantage to ovarian cancer progression. There is evidence that the existence of four independent amplified regions within 11q13 in breast cancer may provide potential coselection and synergistic role of different amplified genes (13). Using interphase FISH technique, Tanner *et al.* (14) found three frequently coamplified regions at 20q11, 20q12, and 20q13.2 in breast cancer. Several candidate oncogenes have been considered as the biological target of amplification events, including *ASC-2* (*AIB 3*) at 20q11 (15), *AIB1* at 20q12 (16), *ZNF217* and *NABC1* at 20q13.2 (17).

As expected, *AKT2* was amplified in all four of the ovarian cancer cell lines and mapped in the overlapping MAR2. *AKT2* is a serine-threonine kinase gene and frequently amplified in ovarian cancer (5). The *PI3K/AKT2* pathway has been demonstrated to be important in malignant transformation (18). Another interesting candidate oncogene in MAR2 is *SEI-1*. Amplification and overexpression of *SEI-1* has been demonstrated by Southern and Northern blot analyses. *SEI-1* was recently isolated by yeast two-hybrid screening using $p16^{INK4a}$ as bait (19). The *SEI-1* gene encodes a M_r 34,000 protein ($p34^{SEI-1}$), a CDK4-binding protein, which renders the activity of *cyclin D/CDK4* resistant to the inhibitory effect of $p16^{INK4a}$ (19). It is well known that the tumor-suppressing function of $p16^{INK4a}$ is through its binding to, and subsequent inhibition of, *cyclin D 1/CDK4* kinase activity. Another study found that $p34^{SEI-1}$ (also named *TRIP-Br1*) interacts with *KRIP-1* (*TIF1β*) and functionally contacts *DP-1*, stimulating *E2F/DP-1* transcriptional activity (20). These features suggest that *SEI-1* is a candidate oncogene, and that its amplification may play an important role in the development and progression of ovarian cancer. Further study of genes within these two MARs may lead to the isolation of the oncogene(s) that is the biological target of the amplification event at 19q13.1 in ovarian cancer. In addition, the results in this study provide a useful basis for future studies of this region for understanding the molecular mechanisms of gene amplification and amplicon evolution in solid tumors.

References

1. Lynch, H. T., Casey, M. J., Lynch, J., White, T. E., and Godwin, A. K. Genetics and ovarian carcinoma. Semin. Oncol., *25:* 265–280, 1998.
2. Iwabuchi, H., Sakamoto, M., Sakunaga, H., Ma, Y. Y., Carcangiu, M. L., Pinkel, D., Yang-Feng, T. L., and Gray, J. W. Genetic analysis of benign, low-grade, and high-grade ovarian tumors. Cancer Res., *55:* 6172–6180, 1995.
3. Sham, J. S. T., Tang, T. C-M., Fang, Y., Sun L., Qin L-X., Xie, D., and Guan X-Y. Recurrent chromosome alterations in primary ovarian carcinoma of Chinese women. Cancer Genet. Cytogenet., *133:* 39–44, 2002.
4. Guan, X-Y., Cargile, C. B., Anzick, S. L., Thompson, F. H., Meltzer, P. S., Bittner, M. L., Taetle, R., McGill, J. R., and Trent, J. M. Chromosome microdissection identifies cryptic sites of DNA sequence amplification in human ovarian carcinoma. Cancer Res., *55:* 3380–3385, 1995.
5. Cheng, J. Q., Godwin, A. K., Bellacosa, A., Taguchi, T., Frank, T. F., Hamilton, T. C., Tsichlis, P. N., and Testa, J. R. AKT2, a putative oncogene encoding a member of subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc. Natl. Acad. Sci. USA, *89:* 9267–9271, 1992.
6. Wunder, J. S., Eppert, K., Burrow, S. R., Gokgoz, N., Bell, R. S., Andrulis, I. L., and Gogkoz. N. Co-amplification and overexpression of CDK4. SAS and MDM2 occurs frequently in human parosteal osteosarcomas. Oncogene, *18:* 783–788, 1999.
7. Guan, X. Y., Trent, J. M., and Meltzer, P. S. Generation of band-specific painting probes from a single microdissected chromosome. Hum. Mol. Genet., *2:* 1117–1121, 1993.
8. Kononen, J., Bubendorf, L., Kallioniemi, A., Barlund, M., Schraml, P., Leighton, S., Torhorst, J., Mihatsch, M. J., Sauter, G., and Kallioniemi, O. P. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat. Med., *4:* 844–847, 1998.
9. van Dekken, H., Alers, J. C., Riegman, P. H., Rosenberg, C., Tilanus, H. W., and Vissers, K. Molecular cytogenetic evaluation of gastric cardia adenocarcinoma and precursor lesions. Am. J. Pathol., *158:* 1961–1967, 2001.
10. Hoglund, M., Gorunova, L., Andren-Sandberg, A., Dawiskiba, S., Mitelman, F., and Johansson, B. Cytogenetic and fluorescence *in situ* hybridization analyses of chromosome 19 aberrations in pancreatic carcinomas: frequent loss of 19p13.3 and gain of 19q13.1–13.2. Genes Chromosomes Cancer, *21:* 8–16, 1998.
11. Guan, X-Y., Meltzer, P. S., Dalton, W. S., *Trent, J.* M. Identification of cryptic sites of DNA sequence amplification in human breast cancer by chromosome microdissection. Nat. Genet., *8:* 155–161, 1994.
12. Schneider, S., Hiemstra, J., Zehnbauer, B., Taillon-Miller, P., Paslier, D., Vogelstein, B., and Brodeur, G. Isolation and structural analysis of a 1.2-megabase N-myc amplicon from a human neuroblastoma. Mol. Cell. Biol., *12:* 5563–5570, 1992.
13. Karlseder, J., Zeillinger, R., Schneeberger, C., Czerwenka, K., Speiser, P., Kubista, E., Birnbaum, D., Gaudray, P., and Theillet, C. Patterns of DNA amplification at band q13 of chromosome 11 in human breast cancer. Genes Chromosomes Cancer, *9:* 42–48, 1994.
14. Tanner, M. M., Tirkkonen, M., Kallioniemi, A., Isola, J., Kuukasjarvi, T., Collins, C., Kowbel, D., Guan, X-Y., Trent, J., Gray, J. W., Meltzer, P., and Kallioniemi, O-P. Independent amplification and frequent co-amplification of three nonsyntenic regions on the long arm of chromosome 20 in human breast cancer. Cancer Res., *56:* 3441–3445, 1996.

15. Lee, S. K., Anzick, S. L., Choi, J. E., Bubendorf, L., Guan, X-Y., Jung, Y. K., Kallioniemi, O. P., Kononen, J., Trent, J. M., Azorsa, D., Jhun, B. H., Cheong, J. H., Lee, Y. C., Meltzer, P. S., and Lee, J. W. A Nuclear Factor. ASC-2, as a cancer-amplified transcriptional coactivator essential for ligand-dependent transactivation by nuclear receptors *in vivo*. J. Biol. Chem., *274:* 34283–34293, 1999.
16. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. AIB1, a novel estrogen receptor co-activator amplified in breast and ovarian cancer. Science (Wash. DC), *277:* 965–968, 1997.
17. Collins, C., Rommens, J. M., Kowbel, D., Godfrey, T., Tanner, M., Hwang, S. I., Polikoff, D., Nonet, G., Cochran, J., Myambo, K., Jay, K. E., Froula, J., Cloutier, T., Kuo, W. L., Yaswen, P., Dairkee, S., Giovanola, J., Hutchinson, G. B., Isola, J., Kallioniemi, O. P., Palazzolo, M., Martin, C., Ericsson, C., Pinkel, D., Albertson, D., Li, W-B., and Gray, J. W. Positional cloning of *ZNF217* and *NABC1*: genes amplified at 20q13.2 and overexpressed in breast carcinoma. Proc. Natl. Acad. Sci. USA, *95:* 8703–8708, 1998.
18. Jiang, K., Coppola, D., Crespo, N. C., Nicosia, S. V., Hamilton, A. D., Sebti, S. M., and Cheng, J. Q. The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. Mol. Cell. Biol., *20:* 139–148, 2000.
19. Sugimoto, M., Nakamura, T., Ohtani, N., Hampson, L., Hampson, I. N., Shimamoto, A., Furuichi, Y., Okumura, K., Niwa, S., Taya, Y., and Hara, E. Regulation of CDK4 activity by a novel CDK4-binding protein, p34(SEI-1). Genes Dev., *13:* 3027–3033, 1999.
20. Hsu, S. I., Yang, C. M., Sim, K. G., Hentschel, D. M., O'Leary, E., and Bonventre, J. V. TRIP-Br: a novel family of PHD zinc finger- and bromodomain-interacting proteins that regulate the transcriptional activity of E2F-1/DP-1. EMBO J., *20:* 2273–2285, 2001.